



Electron pathways involved in H₂-metabolism in the green alga *Scenedesmus obliquus*

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Abstract

The green alga *Scenedesmus obliquus* is capable of both uptake and production of H₂ after anaerobic adaptation (photoreduction of CO₂ or photohydrogen production). The essential enzyme for H₂-metabolism is a NiFe-hydrogenase with a [2Fe–2S]-ferredoxin as its natural redox partner. Western blot analysis showed that the hydrogenase is constitutively expressed. The *K_m* values were 79.5 μM and 12.5 μM, determined with ferredoxin and H₂, respectively, as electron donor for the hydrogenase. In vitro, NADP⁺ was reduced by H₂ in the presence of the hydrogenase, the ferredoxin and a ferredoxin–NADP reductase. From these results and considerations on the stoichiometry we propose that this light-independent electron transfer is part of the photoreduction of CO₂ in vivo. For ATP synthesis, necessary for the photoreduction of CO₂, light-dependent cyclic electron transfer around Photosystem (PS) I accompanies this ‘dark reaction’. PS II fluorescence data suggest that (a) in *S. obliquus* H₂-reduction might function as the anaerobic counterpart of the O₂-dependent Mehler reaction, and (b) the presence of either a ferredoxin quinone-reductase or NAD(P)-dehydrogenase (complex I) in *S. obliquus* chloroplasts. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Ferredoxin; Green alga; Hydrogenase; Mehler reaction; Photosynthesis

1. Introduction

Hydrogenases are well-known redox enzymes cat-

alyzing the reversible oxidation of molecular H₂. While hydrogenases are widespread among prokaryotes, their occurrence in eukaryotes is restricted to a small group of organisms. Some anaerobic protozoa and chytridiomycetes are reported to produce H₂ using hydrogenosomes in a manner similar to anaerobic and fermentative bacteria [1]. In addition, a number of species with hydrogenase activity have been identified in chlorophytes, phaeophytes and rhodophytes [2]. Most intensely the hydrogenases and the H₂-metabolism of the green algae *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* have been studied (for review see [3]). After anaerobic adaptation and under high partial H₂-pressure *S. obliquus* shows a light-dependent uptake of H₂, known as photoreduction of CO₂. Under low partial

Abbreviations: DBMIB, 2,5-dibrom-3-methyl-6-isopropyl-*p*-benzoquinone; EPR, electron spin resonance; FNR, ferredoxin NADP-reductase; FQR, ferredoxin quinone-reductase; NDH, NAD(P)-dehydrogenase (complex I); PAM, pulse amplitude modulation; PQ, plastoquinone; PS, Photosystem; PVDF, polyvinylidene difluoride; SDS-PAGE, denaturing polyacrylamide gel electrophoresis

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H₂-pressure hydrogen is evolved upon illumination, a process called photohydrogen production. Under autotrophic conditions photoproduction of H₂ depends on both PS II and I, whereas photoreduction depends on PS I only. From *S. obliquus* a NiFe-hydrogenase has been purified and characterized [4,5]. In this alga, the enzyme activity is regulated by its redox environment, particularly by plastidic thioredoxin *f* [6].

A comparison of the characteristics of algae with and without hydrogenase activity clearly revealed differences in their behaviour under anaerobiosis ([7] and references therein). In particular, it was shown that the photosynthetic electron transport chain is affected. However, neither the mechanism of electron transfer nor its physiological function is well understood. In this communication we discuss the electron transfer reactions involved in H₂-metabolism and propose a new model of H₂-metabolism in the green alga *S. obliquus*.

2. Materials and methods

All chemicals and reagents were of analytical grade and purchased from either Merck (Darmstadt, Germany) or Sigma (Munich, Germany). Antisera against the large, active site containing subunit of the reversible NiFe-hydrogenase from *Synechocystis* sp. PCC 6803 (HoxH) and *Bradyrhizobium japonicum* and against the Fe-hydrogenase from *Trichomonas vaginalis*, were a generous gift of K. Steinmüller (University of Düsseldorf, Germany), D.J. Arp and L.A. Sayavedra-Soto (Corvallis, OR, USA) and P. Johnson (Los Angeles, CA, USA), respectively. Cells of the wild-type strain of the green alga *S. obliquus* strain D₃ [8] were grown autotrophically as described earlier [9]. Heterotrophic growth and anaerobic adaptation were performed as described by Schnackenberg et al. [4]. The optical densities of algae suspensions were measured with culture medium as reference at 436 nm and corrected for turbidity by subtracting the absorption at 720 nm using a Shimadzu MPS-2000 spectrophotometer (Shimadzu, Kyoto, Japan) [10].

Protein was quantified by the method of Bradford [11], using bovine serum albumin solutions as calibration standards. The molecular masses of protein

and their purity were determined by SDS-PAGE (15%, w/v) [12] and subsequent silver staining [13]. Semi-dry Western blotting on nitrocellulose membranes was performed employing the standard procedure described in [14]. Antibodies were visualized using the alkaline-phosphatase conjugate second antibody system [15].

Hydrogenase was partially purified under anaerobic conditions by the modified procedure of Schnackenberg et al. [4,6]. Ferredoxin was purified by non-denaturing PAGE in analogy to the purification procedure described for cytochrome *c*₆ [16]. Ferredoxin concentrations were determined spectrophotometrically using the molar extinction coefficient of $\epsilon_{420} = 9.7 \text{ mM}^{-1} \text{ cm}^{-1}$ [17].

H₂-Evolution was assayed polarographically in oxygen-free Tris-HCl buffer (50 mM, pH 7.5) containing additionally 15% (v/v) glycerol, 10 vol.% H₂ in N₂, and 10 mM Na-dithionite using a Clark-type electrode [5]. Reduced methylviologen (10 mM) was used as an artificial electron donor in the in vitro experiments. For the determination of the *K*_m for ferredoxin, varying amounts of this compound isolated from *S. obliquus* were used instead of methylviologen. H₂-Uptake was assayed spectrophotometrically in gas-tight 1 ml cuvettes in the absence of oxygen. The H₂-uptake reaction was started by adding 10 mM oxidized methylviologen. All components were dissolved in the above Tris-HCl buffer. Different concentrations of H₂-gas were employed for the *K*_m determinations. The rates of H₂-oxidation were measured by following methylviologen reduction at 578 nm ($\epsilon_{578} = 9700 \text{ cm}^2/\text{mmol}$). The reduction of NADP⁺ was followed spectrophotometrically at 340 nm. The assays contained 0.5 U/ml hydrogenase preparation, 0.05 U/ml spinach FNR, 5 mM NADP⁺ and 4 μM ferredoxin from either *S. obliquus* or spinach. All components were dissolved in the above Tris-HCl buffer.

For protein sequencing, ferredoxin was isolated by SDS-PAGE (15%, w/v) and transferred onto Immobilon PVDF membranes (Millipore, Eschborn, Germany). After staining with Coomassie blue R-250, ferredoxin was cut off and used directly for N-terminal sequencing. Automated Edman sequencing was performed with an Applied Biosystems pulsed-liquid-phase sequencer (Model 471A, Foster City, CA).

EPR spectra were recorded with an ESP 300E

spectrometer equipped with a X-band microwave bridge ER 041 MR and a rectangular cavity. Cooling of the sample was accomplished with a continuous-flow helium cryostat ESR 900. All EPR equipment was purchased from Bruker, Oxford, UK. The reduction of ferredoxin was achieved by the addition of Na-dithionite until the color of the solution changed from brown-orange to dark brown. Samples were frozen in liquid N₂ in an EPR-cuvette (707SQ, Spin-techn, Remshalden, Germany). The measurements were performed at 18 K, 10 mW microwave power, 9.102 GHz microwave frequency, 12.5 kHz modulation frequency and 9 G modulation amplitude.

PS II fluorescence measurements were performed using 2-day-old autotrophically grown cultures of *S. obliquus*. The cells were suspended in culture medium and diluted to an optical density of 0.4 at 436 nm (see above). For the adaptation to different gas atmospheres the cell suspensions were flushed with air, N₂ or H₂ for 2 h in the dark. The fluorescence yield was measured with a PAM-fluorometer (Model 101, Walz, Effeltrich, Germany) applying a continuous flow of the respective gas to the sample [10,18,19]. Before the onset of the actinic illumination, the dark F_0 -levels (fully oxidized Q_A) were monitored by exposing the algae to a weak modulated measuring light ($< 0.1 \mu\text{E m}^{-2} \text{s}^{-1}$, 650 nm, 1.6 kHz). F_m -levels (fully reduced Q_A) were determined by flashing the samples with a 500-ms pulse of saturating white light ($12\,000 \mu\text{E m}^{-2} \text{s}^{-1}$). After the onset of the actinic white light ($190 \mu\text{E m}^{-2} \text{s}^{-1}$), saturation pulses were applied to the algae every 5 s to determine F_m' . On this basis the yield of the photochemical electron transport was estimated by using the equation $\Phi_P = (F_m' - F_{ss})/F_m'$ (Genty-coefficient), where F_m' is the fluorescence yield of a reduced Q_A-pool and F_{ss} the fluorescence in the presence of actinic light [10,20]. Where indicated, PS I was activated by pulses (2 s) of far-red light (714 nm, DIL filter, Schott, Mainz, Germany).

3. Results

3.1. Characterization of the hydrogenase and the ferredoxin

Western blot analysis of the partially purified hy-

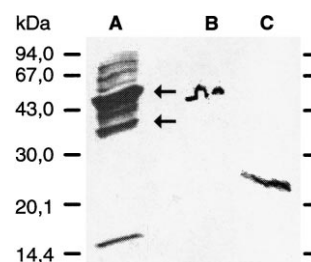


Fig. 1. (A) SDS-PAGE of a partially purified hydrogenase preparation used for the in vitro experiments. The arrows indicate the 36 and 55 kDa subunit of the NiFe-hydrogenase from *S. obliquus*, respectively. (B) Western blot analysis of A, performed with an antiserum raised against the large subunit of the NiFe-hydrogenase from *Synechocystis* PCC 6803. (C) SDS-PAGE of purified *S. obliquus* ferredoxin.

drogenase from *S. obliquus* (Fig. 1A) using an antiserum against the large, active site containing subunit of the reversible NiFe-hydrogenase from *Synechocystis* sp. PCC 6803 [21] showed a clear cross-reaction at 55 kDa (Fig. 1B). This is in agreement with the earlier reported composition of a 36 kDa and a 55 kDa subunit for the *S. obliquus* NiFe-hydrogenase [4]. No cross-reaction was found using a polyclonal antibody against the Fe-hydrogenase from *Trichomonas vagi*-

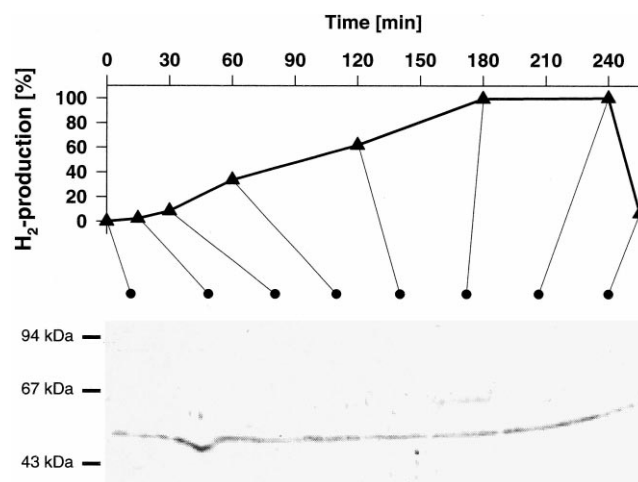


Fig. 2. Western blot detection and in vitro activity of the *S. obliquus* NiFe-hydrogenase at different times of anaerobic adaptation. H₂-Production was tested with the same samples used later for Western blot analysis. An antiserum raised against the large subunit of the NiFe-hydrogenase from *Bradyrhizobium japonicum* was used for immunodetection. After 240 min the adaptation process was stopped by flushing with air. Indicated times (min): 0 (before onset of adaptation), 15, 30, 60, 120, 180, 240 and 255 (15 min after aeration).

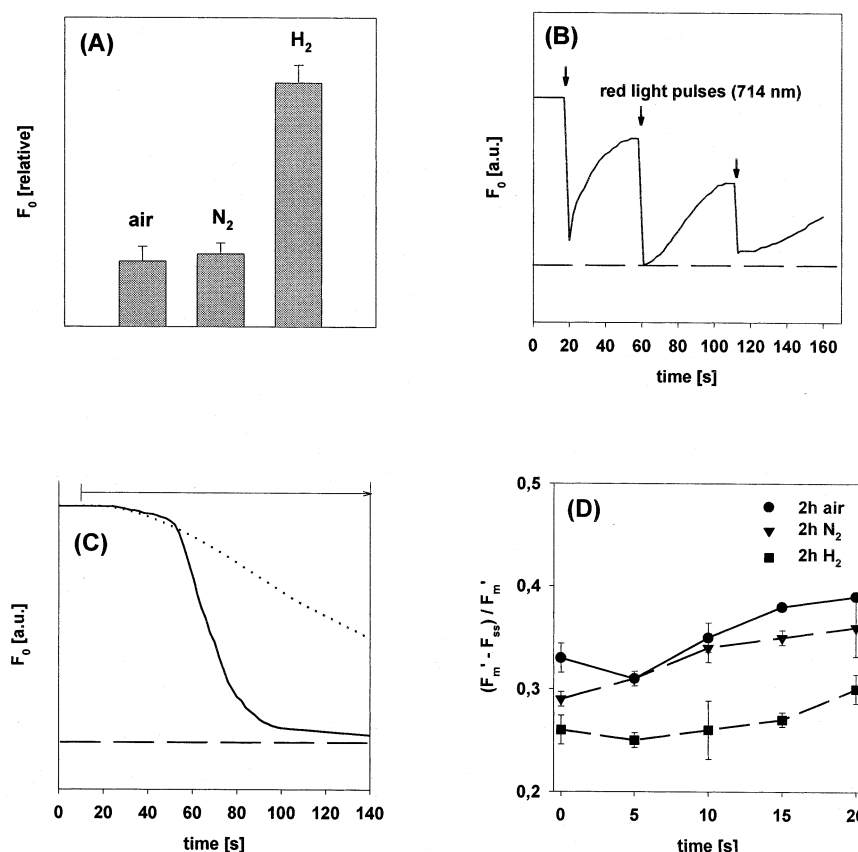


Fig. 3. In vivo PS II fluorescence measurements with *S. obliquus* cells, dark-adapted under the indicated atmosphere. (A) F_0 measurements of air-, N_2 - or H_2 -adapted cells. H_2 -adapted cells were: (B) illuminated with pulses of red light (714 nm) or (C) flushed with either N_2 (—) or air (···) in the dark. F_0 was measured continuously. The broken line indicates F_0 of air- or N_2 -adapted cells. (D) Genty-coefficients measured for air-, N_2 - or H_2 -adapted cells after the onset of actinic light.

*nal*is [22]. For the *S. obliquus* NiFe-hydrogenase K_m values of 79.5 μ M and 12.5 μ M were determined with the homologous ferredoxin and H_2 as electron donor, respectively. The K_m for H_2 corresponds to a partial pressure of 1.7 vol.% H_2 -gas.

Analysis of the purified ferredoxin from *S. obliquus* by SDS-PAGE revealed on silver staining a single band with a molecular mass of about 23 kDa (Fig. 1C). In its oxidized state, the ferredoxin revealed absorption maxima at 325, 415 and 463 nm, respectively. The X-band EPR-spectrum, recorded at 18 K, showed three well-resolved resonances at g values of $g_x = 1.88$, $g_y = 1.96$ and $g_z = 2.05$, a typical signature for [2Fe-2S]-ferredoxins [17]. The first 18 N-terminal amino acids of the purified ferredoxin were determined by micro sequencing to be YKVTLKTPSGTQTIECPE, meaning a very high

identity with the ferredoxin from *S. quadricauda* [23].

3.2. Anaerobic adaptation

To investigate, whether the hydrogenase is activated or synthesized de novo during the anaerobic adaptation process, Western blot analyses were carried out. The amount of hydrogenase protein during the adaptation period was determined by using a polyclonal antiserum against the large subunit of the *Bradyrhizobium japonicum* NiFe-hydrogenase. Although the hydrogenase activity increased during the first 4 h of anaerobic adaptation, the amount of immunodetectable protein showed no variation (Fig. 2). Preparations flushed with O_2 for 15 min after 4 h of anaerobic adaptation, displayed no more hydro-

genase activity, but still showed the same intensity of the cross-reaction at 55 kDa (Fig. 2).

3.3. Reduction of NADP^+ by the hydrogenase/ferredoxin/FNR system

In vitro, H_2 efficiently reduced NADP^+ in the presence of the hydrogenase, ferredoxin and FNR. With rates of 0.6 μmoles reduced NADP^+ per min and ml, homologous ferredoxin from *S. obliquus* was approximately four times more effective than that from spinach. No reduction of NADP^+ was observed in the absence of the hydrogenase.

3.4. PS II fluorescence yields in H_2 -, N_2 -, and air-adapted cells

Cells of *S. obliquus* were flushed with H_2 , N_2 or air in darkness. After 2 h the PS II fluorescence F_0 was measured. In the H_2 -adapted cells F_0 was increased by a factor of 3 compared to N_2 - or air-adapted cells (Fig. 3A). This increase could be reversed by illuminating the cells with pulses of 714 nm red light which activates PS I only (Fig. 3B). This reversion could be measured repeatedly, but with decreasing yield. These data indicate that the primary acceptor of PS II, the PQ-pool, is reduced during the adaptation with H_2 . In a second experiment, H_2 -adapted cells were flushed with either N_2 or air in the dark. As shown in Fig. 3C, F_0 began to decrease after about 50 s while flushing with N_2 . After another 30 s F_0 reached the value of air-adapted cells. Flushing with air led to a significantly slower decrease in F_0 (Fig. 3C). Conclusively, on flushing of the cells with N_2 the PQ-pool became oxidized by the involvement of the hydrogenase. In air-flushed cells, O_2 inhibited hydrogenase activity immediately and PQ-oxidation took place more slowly. Finally, H_2 -, N_2 - and air-adapted cells were illuminated with white actinic light and the Genty-coefficient determined by measuring F_m' and F_{ss} ever 5 s for 20 s (Fig. 3D). Air- and N_2 -adapted cells showed similar and normal fluorescence behavior, indicating a steady electron flow through both photosystems. In H_2 -adapted cells the yield of PS II fluorescence was higher, resulting in a decreased Genty-coefficient. The same effect was observed with N_2 -adapted algae, which were incubated with H_2 -gas for 10 min in darkness. After

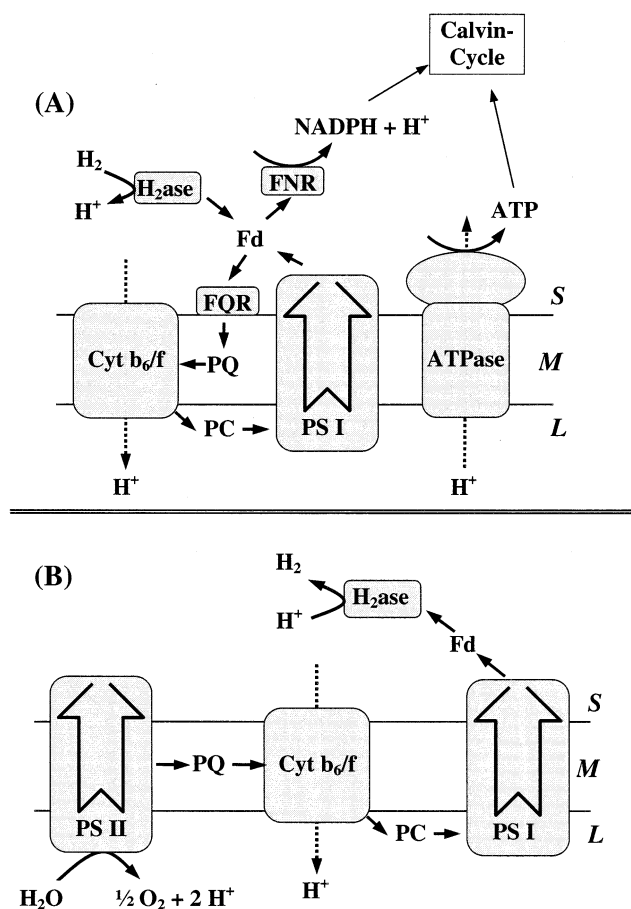


Fig. 4. Model for the H_2 -metabolism in *S. obliquus*: (A) Photo-reduction of CO_2 . Light-independent NADP^+ -reduction by H_2 and cyclic electron flow around PS I with FQR-participation is shown (see text). (B) Photohydrogen production. S, stroma; M, thylakoid membrane; L, thylakoid lumen; H_2ase , hydrogenase; Fd, ferredoxin; Cyt b_6/f , cytochrome b_6/f -complex; PC, plastocyanin; ATPase, ATP synthase; dotted lines, proton translocation; bold straight lines, electron transfer.

the onset of illumination the fluorescence yield exactly matched the curve of H_2 -adapted cells. This indicates that the photosynthetic electron flow was highly impaired.

4. Discussion

Scenedesmus obliquus NiFe-hydrogenase uses ferredoxin as redox partner. This result confirms earlier findings that the NiFe-hydrogenase is involved in light-dependent H_2 -metabolism [3–6]. K_m values for H_2 -evolution and -oxidation, 79.5 and 12.5 μM , respectively, are within the range found for bacterial

NiFe-hydrogenases [24]. Surprisingly, the enzyme is constitutively expressed in *S. obliquus* and its activity independent of its concentration (Fig. 2). Previously, we could show that the redox environment has a regulatory effect on the NiFe-hydrogenase in *S. obliquus* [6]. We conclude that the anaerobic adaptation procedure leads to an activation of the constitutively expressed hydrogenase by changes of the chloroplast redox milieu.

4.1. Electron transfer reactions involved in photoreduction of CO₂

For the photoreduction of CO₂ in algae it is commonly assumed that the electrons derived from the hydrogenase mediated H₂-oxidation enter the PQ-pool [3,7]. This hypothesis is further supported by the fact that the above reaction is inhibited by DBMIB [25]. The ability of ferredoxin to interact with the hydrogenase from *S. obliquus* implies that the electrons from H₂-oxidation can directly enter the Calvin cycle *via* an FNR-mediated NADP⁺-reduction in vivo. Indeed, we could demonstrate this reaction in vitro. Hence, the main function of PS I would be the generation of ATP required for the photoreduction of CO₂ by cyclic electron flow (Fig. 4A). The involvement of cyclic electron flow for photoreduction of CO₂ becomes obvious from stoichiometric calculations. The molar uptake ratio for CO₂/H₂ is 1:2, which exactly meets the CO₂/NADPH requirement of the Calvin cycle [26]. According to the generally accepted interpretation, all electrons deriving from H₂-oxidation directly enter the PQ-pool. After being transferred through the cytochrome *b₆f*-complex and PS I the electrons would then reduce 1 mole of NADP⁺ per mole oxidized H₂. Additionally, 4–6 mole protons per mole H₂ would then be translocated into the thylakoid lumen by the action of the cytochrome *b₆f*-complex [27]. Stromal H₂-oxidation releases 2 moles of protons per mole H₂ into the chloroplast stroma. Hence, only 2–4 moles of protons would be available as proton motive force to drive ATP-synthesis yielding an NADPH/ATP ratio of 1: ≤ 1 [28]. However, the Calvin cycle requires an NADPH/ATP ratio of 2:3. The only reasonable source for an additional ATP-synthesis is the cyclic photophosphorylation *via* PS I. Thus we conclude that PS I activity is only necessary for the additional

ATP-formation, whereas NADPH is provided by a light-independent, direct pathway from H₂ via the hydrogenase, ferredoxin, and the FNR to NADP⁺ (Fig. 4A). However, it should be noted, that the photoreduction of CO₂ requires high partial pressure of H₂. Thus it is doubtful that this pathway plays a significant role in nature. This assumption is supported by previous findings that the hydrogenase activity in *S. obliquus* is inhibited in the presence of reduced thioredoxin [6]. This finding might also explain the requirement of a high partial H₂-pressure, since reduced thioredoxin activates the Calvin cycle enzymes, but inhibits the hydrogenase activity at the same time.

4.2. Indications for a FQR or NDH-complex in chloroplasts of *S. obliquus*

Anaerobically adapted cells of *S. obliquus* consume considerable amounts of H₂-gas in the dark [26]. Presently, it is unknown, which compounds within the cells serve as electron acceptors in this reaction. From PS II fluorescence measurements we assume that the PQ-pool is the electron sink (Fig. 3A–C). But how then do the electrons from H₂-oxidation enter the PQ-pool? We propose that first the electrons are transferred to ferredoxin and subsequently enter the PQ-pool via either FQR or NDH. We propose that the cytochrome *b₆f*-complex does not participate in this electron flow to PQ, as this pathway was shown to be very unlikely in algae [27]. Algal FQR could not be identified on the molecular level yet, but there are several experimental data in favor of its existence [27,29]. The NDH pathway would require the involvement of FNR and the action of a transhydrogenase at the cost of 1 ATP per 2 electrons. Indeed, NADH was reported to be an effective electron donor for the photosynthetic electron transport chain in *Chlamydomonas reinhardtii*. Recently, the NADH-dehydrogenase was partially purified from this organism [30]. We thus propose that the chloroplasts of *S. obliquus* possess either an NDH-complex or an FQR-activity.

4.3. The physiological role of photohydrogen production

For *S. obliquus* it was shown that both photosys-

tems participate in photohydrogen production under autotrophic conditions [31]. With ferredoxin as natural electron donor for the hydrogenase, it thus accepts electrons from PS I (Fig. 4B). Facing anaerobic stress caused by darkness and respiration or environmental conditions, this reaction would enable algae to release excess electrons from the linear photosynthetic electron transport chain during a switch from dark to light, as proposed for cyanobacteria [32]. It has been shown previously, that the O₂-dependent Mehler reaction [33] plays a decisive role for most of the non-photochemical quenching, when CO₂-assimilation is limited [34]. Furthermore, it was shown that photohydrogen production in *C. reinhardtii* increases, when CO₂-concentration decreases [35]. We therefore propose that the Mehler reaction can be replaced by photohydrogen production under anaerobic conditions. Measurements of the PS II fluorescence of anaerobically adapted cells of *S. obliquus* in vivo support this hypothesis. As compared to air-adapted cells, in N₂- and H₂-adapted cells electrons would be expected to queue in the light reaction, since the respective gases replace CO₂. Thus, when light is turned on, the electrons from the photosynthetic electron transport chain cannot be used for CO₂-reduction. However, N₂-adapted cells can still cope with these conditions. Their PS II fluorescence behavior resembles that of aerobic cells (Fig. 3D). We propose that under these conditions the algae are able to maintain the electron flux through the photosystems by releasing excess electrons via the reduction of protons (Fig. 4B). However, in H₂-adapted cells, or N₂-adapted cells facing a highly enriched H₂-atmosphere for some minutes, the 'H₂-valve' is locked. The H₂-evolving reaction of the hydrogenase is inhibited by the high partial H₂-pressure (product inhibition) which leads to the decrease of the Genty coefficient (Fig. 3D). Whether the regulatory function of *S. obliquus* NiFe-hydrogenase is a general feature in hydrogenase containing algae remains to be evaluated.

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